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13. ABSTRACT *(Maximum 200 words)*

An observation was made that the mRNAs for two clones, #41 and #44, were rapidly repressed by estrogen. Clone #44 shared sequence homology with members of the multidrug resistance-associated protein (MRP) gene family. This raised the question of whether the MDR phenotype in breast cancer may in part be due to the loss of repression of MRP by estrogen when TAM is administered. The goals of this past year were to complete the identification of #41 and #44, to ascertain whether they are homologues of MRP, to verify their regulatio by estrogen, and to begin testing whether the human MRP gene may also be regulated by estrogen. Significant progress has been made on most of these goals and a manuscript is in preparation on this work. In particular, we have shown that #44 is the chick orthologue of the human MRP1 gene, we have cloned an additional 1363 bp of #41 but data bases show no significant homologies with other cloned genes, the half lives of repression of the mRNAs for the two clones have been determined, and preliminary data suggest that the regulation of the chick MRP gene is probably post-transcriptiona.

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FOREWORD

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INTRODUCTION

The overall goal of our research has been to investigate the regulation of eucaryotic gene expression by steroid hormones. Through the use of differential display, we discovered that two mRNAs are rapidly repressed by estrogen in chick oviduct. During the past year, we've discovered that one of these shares 73% identity at the amino acid level with the human multidrug resistance-associated protein (hMRP). This protein is a member of the ATP-binding cassette (ABC) transmembrane transporter family. As members of this family are implicated in the development of resistance to therapeutic drugs, the ability to regulate the amount of MRP by estrogen selectively in mammary cells may provide a novel means of enhancing the efficacy of chemical interventions for breast cancer.

The ABC Transmembrane Transporter Family: The ABC transporter family is ubiquitous from bacteria to man (1) and contains over 50 members (2). In vertebrates, the ABC transporters can be divided into four subfamilies: 1) the P-glycoproteins (Pgp) encoded by the multidrug resistance (*mdr*) genes, 2) the TAP proteins encoded by the major histocompatibility complex, 3) the peroxisomal membrane proteins, and 4) the cystic fibrosis transmembrane conductance regulators (CTFR) (2). Thus, these transporters are associated with clinical problems such as the multidrug resistance (MDR) of cancers, antigen presentation, and cystic fibrosis. The transporters utilize the energy of ATP to pump a wide variety of compounds including drugs, amino acids, steroid hormones, sugars, inorganic ions, and peptides into or out of cells (2). The transporters are structurally related, and most contain two nucleotide-binding domains and two hydrophobic transmembrane domains, each containing six transmembrane helices.

The multidrug resistance transporters are of particular importance as they play a key role in the development of resistance to chemotherapeutic drugs. There are two human *mdr* genes (*mdr*1 and *mdr*2) but only *mdr*1 confers resistance to drugs when overexpressed (3). The normal cellular function of the protein product of the *mdr*1 gene, Pgp, is unclear, but its high abundance in adrenal cortex and gravid uterus suggest that at least one of its roles may involve the secretion of steroid hormones (4). The MRP branch of the multidrug transporters has six cloned members (*MRP1-6*) thus far (5, 6) four of which (*MRP3-6*) have been cloned since this grant was originally written. The best characterized one is encoded by the *MRP1* gene (5). Structurally, MRP is more closely related to the CTFR family members than it is to Pgp (5). MRP1 is an organic anion pump referred to as a glutathione-S-X or GS-X pump. MRP1 is able to transport both anionic drug conjugates and unmodified cancer drugs. Therefore, like Pgp, overexpression of MRP increases resistance to a wide spectrum of natural product drugs (7, 8). Furthermore, multidrug resistance in a number of cancers has been ascribed to overexpression of MRP (9, 10 and references therein). For example, expression of MRP was observed in 47 out of 55 (87%) human cancer cell lines even though they were not selected for drug resistance (11). Of particular interest to this proposal, expression of MRP mRNA

is 10-fold higher in a human breast carcinoma cell line that was selected for resistance to etoposide (VP-16) (10).

Considerable evidence has accumulated to suggest a complex relationship between some ABC transporters and steroid hormones. For example, some transport steroids out of cells (12 - 17). This raises the possibility that members of the ABC family modulate the efficacy of steroids by altering their intracellular concentration and hence their availability to the intracellular receptors. Thus, the sensitivity of particular tissues to steroids may depend in part on the nature and activities of selective ABC transporters. In addition, expression of the *mdr* genes is increased by steroids. Expression of the *mdr*1 gene in mouse uterus is enhanced after two days of treatment with estrogen and progesterone (18, 19), although estrogen by itself is ineffective (18). As the mdr mRNA increases over 2,000-fold and the effect on transcription is only 2- to 3-fold, this effect of steroids is predominantly due to an increase in the stability of the mRNA (19). No relationship between any *MRP* genes or protein and steroid hormones has yet been described.

Interest in the MRP family and its role in cancer is burgeoning. A Medline search indicates that there were 167 papers in 1997 and 1998 on MRP. However, few address the normal regulation of the *MRP* genes, and none describe regulation by steroid hormones. Thus, our studies are very topical and of considerable importance to understanding the contribution of these proteins to the MDR phenotype.

Through the use of differential display, we have been cloning genes that appear to be primary targets of the estrogen receptor in chick oviduct. Two such clones were identified whose expression is rapidly repressed by estrogen, clones #41 and #44. During the past year we have gathered data that attest to the fact that clone #44 is the chick orthologue (*chMRP*) of the *MRP1* gene (Appendix A, Fig. 1 and 2). We suspect that #41 may also encode a member of this family of proteins for the following reasons. Both clones detect mRNAs of similar size, about 6.6 kb (*chMRP*) and 7.5 kb (#41). Also, the kinetics of mRNA loss in response to estrogen are similar (Appendix A, Fig. 4 and Appendix B, Figs. 3 and 4). However, there are some differences that suggest that they are encoded by different genes. For example, while the mRNAs are distributed comparably in several tissues, clone #41 mRNA is high in oviduct but low in lung while chMRP mRNA is higher in lung than in oviduct (Appendix A, Fig. 5 and Appendix B, Figs. 5 and 6).

HYPOTHESIS/PURPOSE:

The hypothesis under investigation is that estrogen directly represses the transcription of the human *MRP1* gene. The original goals of these studies were to determine whether clone #44 is a chick homologue of one of the human *MRP* genes, whether clone #41 represents another yet unidentified member of the multidrug resistance transporter family, whether expression of the human *MRP* homologue is repressed by estrogen, and whether that repression represents an effect on transcription or mRNA stability. Our broader hypothesis is that at least some estrogen-independent breast cancer cells become resistant to

chemotherapeutic drugs when estrogen receptor is no longer capable of repressing expression of the *MRP1* gene. These proposed studies will provide the foundation upon which to design experiments that are directly applicable to designing innovative therapies for breast cancer based on manipulating the expression of the *MRP1* gene.

BODY

TECHNICAL OBJECTIVES:

Specific Aim I: To characterize clones #44 an #41 to determine whether they are homologues of the human *MRP* gene family

Specific Aim II: To determine whether the human *MRP1* gene is regulated by estrogen

Specific Aim III: To determine whether the regulation by estrogen is at the level of transcription, and, if so, to begin to define the critical regulatory mechanisms

RATIONALE, METHODS, AND RESULTS:

Specific Aim I: To characterize clones #44 an #41 to determine whether they are homologues of the human *MRP* gene family

Rationale:
Preliminary results suggested that two members of the *MRP* transporter family have been cloned from chick oviduct. Clone #44 appeared to be the orthologue of human *MRP1*. Based on the tissues in which it is expressed, clone #41 did not appear to be human cMOAT (*MRP2*), the only other *MRP* family member cloned at that time (6). The goal of this Specific Aim was to more completely characterize the chick cDNAs as the basis for extending the observation regarding the regulation by estrogen to the human. As both chick clones were less than 300 bp, the first objective was to obtain longer cDNAs for each so that sequence comparisons could be made.

Cloning and sequencing of a larger cDNA for clone #44:
As described in more detail in Appendix A, a chick cDNA library prepared from estrogen-withdrawn chick oviduct was screened with the radiolabeled 266 bp clone #44. Positive clones were restriction enzyme mapped, sequenced, and aligned. An additional 1332 bp of sequence was obtained and was used to search databases for homologous sequences. This analysis revealed that clone #44 is indeed the chick orthologue of the human *MRP1* gene as the two are 73% identical at the nucleotide level (Appendix A, Fig. 1). Furthermore, alignment of the translated *chMRP* clone with the human and murine MRP1 proteins shows a 73% identity and 79% similarity at the amino acid level

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(Appendix A, Fig. 2). The similarity at both the nucleotide and amino acid levels between the *chMRP* cDNA clone and the human and mouse *MRP* genes for this large a clone indicates that the clone is the chicken orthologue of the *hMP1* gene and is part of the superfamily of ABC transmembrane transporters. Because there is no need to obtain the entire 6.6 kb *chMRP* cDNA for these studies, **this task is now complete.** These data are in the process of being incorporated into a manuscript (Appendix A).

Cloning and sequencing of the cDNA for clone #41:

A chick cDNA library from estrogen-withdrawn chick oviduct (Methods, Appendix A) was screened with the 250 bp radiolabeled differential display product #41. Positive clones were identified, restriction mapped, and sequenced, and an additional 1,363 bp have been obtained (Appendix B, Fig. 1). However, this sequence appears to be entirely within the 3'-untranslated region, and database searches reveal no significant homologies with other genes. Thus, additional 5' sequence must be obtained. Because all of the positive clones within this library have been characterized, 5'-RACE was employed in hopes of generating longer clones. Unfortunately, the products of this PCR reaction give a smear rather than a discrete band on the polyacrylamide gel. This is presumably because the mRNAs are so long, approximately 7.5 and 6.4 kb, that the reverse transcription reaction yields products of various sizes. Thus, upon amplification in the RACE reactions, a smear is obtained. We have therefore turned to making a new estrogen-withdrawn chick oviduct library with the hope that this library will contain longer inserts. Both oligo(dT) and random primers were used so that clones from the 5'-end would be represented in the library. Thus far, both cDNA strands have been synthesized, the linkers have been added, and the resultant cDNAs are ready to insert into the phage arms. Once this library is ready, then it will be screened as before with the 1,363 bp #41 clone. If this library also proves inadequate for obtaining more sequence, then we will purchase a chick heart library from Clontech as heart is one of those tissues that expresses large amounts of #41 (Appendix B, Figs. 5 and 6). We are optimistic that clones will be obtained in the near future that include at least part of the coding sequence so that we can determine whether this is a novel or previously identified clone and ascertain whether it is part of the ABC transporter family. Although, we do not as yet know the identity of #41, we have already undertaken considerable characterization of the regulation of the gene (Appendix B, Figs. 1 and 3 - 6), so once the identity is established, a manuscript can be submitted promptly.

Future directions:

It is unclear whether #41 will prove to encode a new *MRP* family member or, if it does, whether a homologue exists in humans. Similarities in the characteristics of the mRNAs that hybridize to *chMRP* and #41, as mentioned in the *Introduction*, suggest that the two clones are related, but this merely provides circumstantial support. If clone #41 appears to be a novel *MRP* family member based on sequence homology, then experiments will be initiated to

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clone the human homologue. Because the *MRP* gene appears to be expressed in a somewhat tissue-specific manner (Appendix A, Fig. 5), the first step will be to probe a northern blot of various human tissues (already purchased from Clontech for other studies) with a fragment from the chick clone that encompasses one of the conserved nucleotide binding domains. Once a tissue is identified that expresses a reasonable amount of the homologous RNA, then a corresponding human cDNA library will be purchased (Clontech). Any novel clone(s) that appears to be regulated by estrogen as determined in *Specific Aim II* will be pursued. Thus far, the *MRP* gene family has not been well characterized. Therefore, as part of our cloning effort, Southern blots with human DNA will be run to determine roughly how many genes there are that hybridize to *chMRP* and #41. This information will be important for designing a cloning strategy if multiple genes exist, for interpreting the effects of estrogen, and ultimately for designing treatment strategies. Regardless of the results, though, the proposed characterization of both clones should not present any substantial technical difficulties. Analysis of the chick genes will be dropped with regard to this proposal as soon as sufficient information is obtained to move with confidence into the human system. The main priority will continue to be analysis of the regulation of the *MRP* gene and then to characterize clone #41 as time permits and, if appropriate, clone its human estrogen-responsive homologue.

Specific Aim II: **To determine whether the human *MRP* gene is regulated by estrogen**

Rationale:
Efforts over the last few months have shown that clone #44 is the chick orthologue of human *MRP1*. As the chick mRNA rapidly decreases ($t_{1/2}$ = ~45 min) upon injection of estrogen (Appendix A, Fig. 4), this raises the intriguing possibility that the human gene is also regulated by estrogen. This contention is supported by the observation that human MRP mRNA is markedly reduced in estrogen-responsive tissues such as ovary, brain, and liver (20). Additional circumstantial evidence comes from the observations that the human breast cancer MCF-7 cell line typically loses functional estrogen receptors as the cells acquire resistance to drugs (21, 22). The ultimate goal of this specific aim is to determine whether the human *MRP1* gene is regulated by estrogen. If it is, this may explain how at least some estrogen-independent breast cancers become resistant to chemotherapeutic drugs. Because of the difficulties associated with determining whether the *MRP1* gene is regulated by estrogen in humans, these studies will use human breast cancer cell lines, particularly MCF-7 cells.

Demonstration that the chMRP and clone #41 mRNAs are regulated by estrogen.
Prior to analyzing the regulation of the *hMRP1* gene, we wanted to verify that the down-regulation of the differential display chick clone by estrogen was in fact real. Therefore, to confirm that chMRP mRNA is indeed differentially

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expressed in estrogen-stimulated and estrogen-withdrawn chicks, northern blot analysis was carried out using total RNA isolated from chick oviduct (Appendix A, Fig. 3). RNA isolated from withdrawn chick oviduct shows strong hybridization of the *chMRP* DD-PCR probe to an mRNA species of approximately 6.6 kb. In contrast, the stimulated oviduct lane shows a dramatic decrease in the hybridization signal relative to the withdrawn lane.

To determine whether estrogen regulates chMRP mRNA acutely, withdrawn chicks were injected with 17β-estradiol and, at the indicated times, the oviducts were removed and RNA isolated. As shown by northern blot analysis (Appendix A, Figs 4A and 4B), chMRP mRNA is abundant in the estrogen-withdrawn oviduct (time 0 hour) but rapidly decreases by thirty minutes after injection of estrogen. Densitometric analysis of the northern blots indicates that the chMRP mRNA levels decrease by 70% within one hour, to a level equal to that observed with chronic estrogen administration (Appendix A, Figure 4B). These results indicate that the *chMRP* gene is down-regulated by estrogen in chick oviduct and that the decrease in chMRP mRNA occurs acutely, within one hour of estrogen administration.

To ascertain whether chMRP is present in other tissues in the chick, northern blot analysis was used to determine chMRP levels in RNA isolated from estrogen-withdrawn oviduct, liver, brain, heart, kidney, lung, skeletal muscle, and intestine (Appendix A, Figures 5A and 5B). The results demonstrate that chMRP mRNA is most abundant in oviduct, heart, and lung but is also expressed, albeit at about 75% lower levels, in brain, skeletal muscle, kidney, and intestine. chMRP mRNA was not detected on our northern blots in liver. These results are comparable to those found with murine MRP mRNA, which is abundant in lung, heart, kidney, testes, and muscle and is also expressed in very low levels in the liver.

Lastly, we wanted to determine whether the *chMRP* gene is regulated by estrogen in other estrogen-responsive tissues. Various tissues were collected from chicks that were treated with estrogen or withdrawn from estrogen for five days. As shown in Fig. 2 (Appendix B), estrogen has no affect on the amount of chMRP mRNA in heart, kidney, or brain, all of which have estrogen receptor. As expected, no differences were observed in lung or intestine. Thus, the oviduct appears to be the only tissue tested in which the chMRP mRNA is regulated by estrogen. However, there is some evidence that other members of the ABC transmembrane transporter family may be regulated by steroids. Mouse MDR mRNA is increased by estrogen and progesterone in uterine epithelial cells and expression of the cystic fibrosis transmembrane regulator gene is also increased by estrogen in uterine epithelial cells. However, our results are the first to show down-regulation of a member of the ABC transmembrane transporter family by steroids.

We are just beginning the proposed studies to measure the effects of estrogen agonists and antagonists on hMRP mRNA in various estrogen-responsive cell lines. We are in the process of establishing a quantitative, competitive reverse transcription PCR (RT-PCR) reaction with an internal control molecule (23) to more accurately and quickly quantitate the amount of

hMRP mRNA in the cells. Primers have been designed that should amplify the DNA in the third transmembrane domain and that would likely share homology with the human, mouse, and chick genes. However, these primers will not detect mRNAs from other *MRP* gene family members. Conditions are now being worked out to obtain linear and quantitative RT-PCR results. Comparable assays have already been successfully set up in my lab to measure the mRNAs for two transcription factors, the estrogen receptor and δEF1, so we have considerable experience and anticipate no difficulties getting it to work for MRP mRNA. We are also in the process of obtaining the cell lines to use for these experiments.

Although we have not yet identified clone #41, considerable progress has been made in characterizing its regulation by estrogen and tissue distribution (Appendix B, Figures 3 - 6). To determine whether estrogen regulates clone #41 mRNA acutely, withdrawn chicks were injected with 17β-estradiol and, at the indicated times, the oviducts were removed and RNA isolated. As shown by northern blot analysis (Appendix B, Figs. 3 and 4), clone #41 mRNA is abundant in the estrogen-withdrawn oviduct (time 0 hour) but rapidly decreases by one hour after injection of estrogen. Densitometric analysis of the northern blots indicates that the clone #41 mRNA levels decrease by 50% within one hour and by two hours are at a level comparable to that observed with chronic estrogen administration (Appendix B, Figure 4). These results indicate that expression of clone #41 mRNA is down-regulated by estrogen in chick oviduct and that the decrease occurs acutely, within one hour of estrogen administration.

To ascertain whether clone #41 mRNA is present in other tissues in the chick, northern blot analysis was used to determine its levels in RNA isolated from estrogen-withdrawn oviduct, liver, brain, heart, kidney, lung, skeletal muscle, and intestine (Appendix B, Figure 5). The results demonstrate that clone #41 mRNA is most abundant in oviduct, heart, and intestine but is also expressed, albeit at lower levels, in liver, brain, skeletal muscle, kidney, and lung. Thus, clone #41 appears to be ubiquitously expressed, although the amount of mRNA varies considerably from one tissue to another. Because clone #41 detects two bands, one of about 7.5 kb and one about 6.4 kb, we wanted to determine whether one of the mRNAs was preferentially expressed in some tissues. Thus, densitometric analysis was done on the two bands in the northern blot shown in Fig. 5. The results indicate that the two bands appear to be expressed in roughly the same ratios in all tissues tested (Appendix B, Fig. 6). Thus, there is no evidence of tissue-specific alternative splicing or promoter usage.

Future directions:

Once the quantitative RT-PCR assay is ready, the first series of experiments will be to determine whether MRP1 mRNA is repressed in wild type MCF-7 cells by estrogen agonists or affected by estrogen antagonists. The amount of MRP1 mRNA will be determined by quantitative RT-PCR after 0.5, 1, 2, and 4 hours of treatment. If no effect on MRP mRNA(s) is observed with the

estrogen agonists and antagonists, then other human breast cancer cell lines such as ZR-75 or BT474 that retain at least some responses to estrogen will be used (24).

Assuming that the hypothesis is correct and that estrogen rapidly represses expression of the *MRP* gene in MCF-7 cells, then several additional experiments will be performed to obtain information about the mechanisms involved. For example, protein synthesis inhibitors will be added concomitantly with estrogen as a first approach to determining whether the repression is a primary effect of estrogen. Other experiments will include analyzing the recovery time after estrogen withdrawal to ascertain how long repression by estrogen is maintained. Additional experiments will compare the regulation of the *MRP* gene in various derivative cell lines. Thirty percent of the cases of antiestrogen resistance arise even in the presence of ER (25). One possible mechanism is that expression of the *MRP* or related genes (clone #41 homologue) is no longer repressed by ER. Another is that the estrogen antagonists are actually inducing the *MRP* gene. To examine these questions, expression of the *MRP* gene(s) will be determined in LY-2 (25), MCF7/MIII (26), and MCF7/LCCl (26) cells, which are ER positive but antiestrogen resistant. Another line (MCF7/LCC2) is resistant to TAM but not to the steroidal antiestrogens ICI 182,780 or ICI 164,384 (26). By comparing the expression of the *MRP* gene(s) in these and other cell lines that vary in their responsiveness to estrogen agonists and antagonists and in their resistance to drugs, it should be possible to address the question of whether some breast cancers become resistant to mixed estrogen antagonists like TAM because they induce rather than repress expression.

If there is no effect of estrogen agonists or antagonists on *MRP* gene expression in the breast cancer cell lines, then either the gene is regulated differently in human than in chick or some other event in the signaling pathway differs between the cell lines and oviduct cells. To address this, experiments will be done to assess whether the *MRP* gene is regulated by estrogen in the mouse. Estrogen-responsive and non-responsive tissues will be isolated from mice during the different stages of the estrous cycle to determine whether the amount of MRP mRNA correlates with the amount of circulating estrogen. Although the mouse *MRP* gene has not been cloned, Parek and Simpkins (27) used primers to the human gene to measure MRP mRNA in rodent cells. In addition, female mice will be injected with estrogen and the amount of MRP mRNA determined in a number of tissues at 0.5, 1, 2, and 4 hours after injection. If no effect of estrogen is seen on the mouse *MRP* gene or if difficulties are experienced with those experiments, the last approach would be to transfect an ER expression vector into an MCF-7 cell line such as MCF7/VP that expresses MRP (10) to determine whether overexpression of ER will repress *mMRP* gene expression. Because MRP mRNA is so rapidly and extensively repressed by estrogen in the chick, it is anticipated that this will also be the case in human and mouse. At least some of the experimental approaches proposed herein should definitively address that hypothesis.

Specific Aim III: **To determine whether the regulation by estrogen is at the level of transcription, and, if so, to begin to define the critical regulatory mechanisms**

The rapid loss of MRP mRNA in the chick oviduct after treatment with estrogen could reflect an effect on the transcription of the gene, on the stability of the resultant mRNA or both. Although no one has examined the half-life of MRP mRNA, that for mdr mRNA is 30 - 60 minutes (28), which is consistent with the half-life of MRP mRNA observed in chick. To determine whether estrogen is repressing the transcription rate of the *MRP* gene, nuclear run-on assays have been done using nuclei isolated from chick oviduct that had been withdrawn or treated with estrogen. The nuclear run-on assays were performed as we have done before (29). This experiment has been done three times using the complete cDNA clone as the probe, and no signal was seen from the *MRP* cDNA slots despite the fact that signals were seen with two transcription factors, δEF1 and HNF3β (data not shown). This indicates that the level of transcription of the *MRP* gene is very low, even without estrogen. This implies that the level of regulation by estrogen is post-transcriptional, probably at the level of mRNA stability.

To approach this question in another way, oviduct cell cultures have been treated with and without estrogen and with and without actinomycin D, an inhibitor of RNA polymerase. At various times after actinomycin D treatment, the cells were harvested, and the RNA was collected. Once the quantitative RT-PCR assay is available, the amount of MRP RNA present in the various conditions will be determined. This experiment should provide information about whether estrogen is acting at the transcriptional or post-transcriptional levels.

Future directions:

To complement the transcription studies that are in progress, pulse chase experiments (30) in the presence and absence of estrogen with either the breast cancer cells or primary chick oviduct cells will be done to examine the rate of decay of MRP mRNA. By comparing the information supplied by each of these techniques, it will be possible to ascertain whether estrogen is affecting transcriptional or post-transcriptional events.

If the effect of estrogen appears to be primarily at the level of transcription, then experiments will focus on defining the critical regulatory elements. This will involve standard transfection experiments to locate the regulatory elements as well as *in vitro* protein-DNA binding assays to define the relevant nucleotides. Thus far, little is known about the regulatory elements that control transcription of the *MRP1* gene. A 2.2 kb fragment linked to the CAT reporter was used in HL60 cells to define the start site of transcription as well as basal promoter elements (31). For the transfection experiments, a large fragment of 5'-flanking DNA (5 - 8 kb) will be isolated from a human genomic library. A CAT plasmid will be constructed containing this flanking DNA and the promoter region. This plasmid will be transfected into the appropriate human breast cancer cell line and assessed for its repression by estrogen. If

transcription is repressed, serial 5'- and 3'-deletions will be made to define the minimal estrogen response element/unit. This functional unit may not contain a typical ERE as there have been no reports of "negative" EREs. Instead, ER may act through other mechanisms such as antagonizing the effects of a transcriptional activator such as c-Fos (32). Thus, it will be necessary to define the exact nucleotides to which the repressive effects of estrogen map by using *in vitro* DNase I footprinting and gel mobility shift assays to elucidate the mechanisms by which estrogen represses *MRP1* gene expression.

If estrogen represses expression of a reporter construct containing the 5'-flanking region of the *MRP* gene, then the subsequent experiments are straightforward and utilize techniques with which we have considerable expertise (29, 33, 34, 35, 36, 37). However, if no regulation by estrogen is observed, this may be because the regulatory elements are not in the 5'-flanking region. The next step would be to map estrogen-responsive elements in the gene and flanking regions using DNase I hypersensitivity. This approach has been widely used to locate regulatory regions in DNA prior to transfection studies (38) and thus should prove successful in defining a region that is regulated by estrogen. However, we are proposing the initial transfection experiment first because it is relatively rapid, regulatory elements are typically within the 5'-flanking region, and it would not require cloning and mapping a large region of human DNA.

If the effect of estrogen appears to be primarily at the level of mRNA stability, then the analysis of the regulatory mechanisms becomes more complex. However, techniques are now available that make these studies feasible. For example, a plasmid expression system has been developed that allows one to determine which sequences are responsible for the regulated stability (39). Furthermore, RNA-protein binding assays have identified proteins that affect the stability of mRNA, including a binding protein that is induced by estrogen (40). If the regulation of the amount of MRP mRNA is at the level of stability, then experiments will be undertaken using these tools to define the mRNA sequences that are responsible for this effect. This will ultimately be followed by attempts to identify the regulatory protein(s) by RNA-protein binding assays. Although we have no experience with this technique, expert advice is available within our Department.

CONCLUSIONS

In the past year, we have shown conclusively that clone #44 is the chick orthologue of the human *MRP1* gene. Furthermore, we have confirmed that the expression of this gene is down-regulated by estrogen in chick oviduct with a half-life of about 45 minutes. The kinetics of this response in conjunction with the low transcriptional activity of the gene as determined by nuclear run-on assays suggest that the effect of estrogen is post-transcriptional, probably at the level of mRNA stability. In addition, the tissue distribution of the chMRP mRNA has been determined. Interestingly, liver contains no detectable MRP mRNA but all other tissues tested have some. A manuscript describing this work is nearly

complete and only lacks the transcription data (Appendix A). It is anticipated that the work will be submitted to *Endocrinology* before the end of this calendar year.

Efforts to identify clone #41 have been less successful, although over 1,000 additional bp have been cloned and sequenced. Nonetheless, characterization of the regulation of the clone has continued. Expression of the clone #41 mRNA is rapidly repressed by estrogen, with a half live of about one hour. The gene appears to be ubiquitously expressed, although the amount of the mRNA does vary and is especially low in liver.

These experiments confirm our preliminary observations that the mRNAs of one or more members of the *MRP* gene family are rapidly repressed. This thus provides the foundation for more mechanistic studies to determine how this repression occurs and what functional significance it may have for the multidrug resistance phenotype observed in some breast cancers. In the next couple of months, the tools should be in place to completely expand these studies into human breast cancer cells.

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Down-Regulation of the Chick Multidrug Resistance-Associated Protein (*MRP*) Gene by Estrogen

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Running Title: Estrogen regulates the multidrug resistance-associated protein gene

Abstract

Although a number of genes have been identified that are up-regulated by estrogen, few have been discovered that are down-regulated. However, gene repression is a common mode of signal transduction by the nuclear receptors. In hopes of determining whether estrogen can directly down-regulate genes, attempts were made to identifying genes that are direct targets of the estrogen receptor and that are down-regulated by it. Because the development and differentiation of the chick oviduct is dependent upon estrogen, this seemed an appropriate model system for testing this hypothesis. mRNA was isolated from estrogen-treated and estrogen-withdrawn chick oviduct and was subjected to differential display polymerase chain reaction. Surprisingly, one of the products that were down-regulated by estrogen encoded the chick homologue of the human and mouse multidrug resistance-associated protein (*MRP*) gene. The partial chick *MRP* (chMRP) cDNA clone contains 1322 base pairs that shows 73% identity with the human cDNA clone. Translation of the *chMRP* cDNA clone shows 73% amino acid identity with both the human and mouse proteins. The regulation of the *chMRP* gene by estrogen was assessed by northern blot analysis. In the oviduct, estrogen rapidly attenuates the amount of chMRP mRNA. A 50% decrease occurs within thirty minutes of estrogen administration and a 70% decrease by 1 hour, which is comparable to the level observed with chronic repression by estrogen. chMRP mRNA is present in many other tissues including the heart, lung, brain, kidney, skeletal muscle, and intestine but is undetectable in the liver. This study indicates that in estrogen-responsive tissues such as chick oviduct, the regulation of *chMRP* gene expression is controlled by estrogen.

Introduction

Multidrug resistance (MDR) occurs in many different tumor types and presents an obstacle to the treatment of these tumors (Gottesman and Pastan, 1993). The MDR phenotype is characterized by the resistance of the tumor to chemotherapeutic agents that are structurally and mechanistically unrelated. MDR can occur inherently, as in renal and colon carcinomas, or can be acquired during treatment of the tumor by chemotherapeutic agents as in lymphomas and breast carcinomas (Gottesman and Pastan, 1993). The MDR phenotype is derived at least in part to the expression of two genes, *MDR1* which encodes P-glycoprotein and *MRP* which encodes the multidrug resistance-associated protein (MRP) (Cole et al., 1992; Grant et al., 1994; Gros et al., 1986; Ueda et al., 1987). Both P-glycoprotein and MRP belong to the ATP-binding cassette (ABC) transmembrane transporter family and are characterized by multiple membrane spanning domains and nucleotide binding domains (Higgins, 1992). It is thought that MDR is conferred upon tumor cells by P-glycoprotein or MRP through the transport of chemotherapeutic agents out of a cell by way of these transmembrane transporters.

The human *MRP* gene was cloned and characterized from the small-cell lung cancer cell line H69AR and was shown to provide multidrug resistance to transfected cells (Cole et al., 1992; Grant et al., 1994). The amino acid sequence of MRP indicates that it belongs to the superfamily of ABC transmembrane transporter proteins. Members of the ABC transmembrane transporter superfamily are ubiquitous from bacteria to man and transport a variety of molecules including amino acids, sugars, inorganic acids, polysaccharides, peptides, and proteins (Higgins, 1992). While the physiological role of MRP remains largely unknown, it can act as an ATP-dependent transporter of cysteinyl leukotrienes, glutathione disulfide, and steroid conjugates including 17ß-estradiol 17-(ß-D glucuronide) (Leier et al., 1994; Loe et al., 1996).

Estrogen plays a critical role in the differentiation and development of the chick oviduct. The action of estrogen in regulating oviduct differentiation occurs through nuclear estrogen receptors, which regulate either the increase or decrease in transcription of specific genes (Beato, 1989; Tsai and O'Malley, 1994). To further understand the mechanism of estrogen-regulated gene expression in the chick oviduct, identification of primary targets of the estrogen

receptor is necessary. Using differential display PCR (DD-PCR) (Liang and Pardee, 1992; Liang et al., 1994), a fragment of an estrogen down-regulated gene showing homology to the human *MRP* gene was cloned. Investigation into the regulation of chick *MRP* gene expression by estrogen is presented in this report. Understanding the mechanism of estrogen action in regulating *MRP* expression could prove useful in the elucidation of the function of MRP in development and differentiation and possibly provide clues as to its regulation in the treatment of tumors.

Materials and Methods

Animals. Sexually immature female white leghorn chicks were subcutaneously implanted with two 10 mg diethylstilbestrol (DES), a synthetic estrogen, pellets (Hormone Pellet Press, Leawood, Kansas) for 2 weeks. "Stimulated" chicks retained the DES pellets while the pellets were removed from "withdrawn" chicks 5 days prior to use. For acute estrogen stimulation, "withdrawn" chicks were injected in the wing vein with 25 mg/kg 17ß-estradiol (dissolved in propylene glycol). The chicks were then sacrificed at 0.5, 1, 2, and 4 hours.

DD-PCR and cloning of the chMRP cDNA. Oviducts from estrogen-stimulated and -withdrawn chicks were collected and total RNA was isolated using an RNeasy RNA Isolation Kit (Qiagen). DD-PCR was carried out as previously described (Liang and Pardee, 1992; Liang et al., 1994) with modifications. Reverse transcription was carried out using oligo $dT_{11}G$, $dT_{11}C$, and $dT_{11}A$ primers in separate reactions. The PCR was carried out using the original oligo $dT_{11}N$ primer and a single random primer (5'-TGACGTACAC-3'). cDNAs were compared between the DES-stimulated and -withdrawn RNA samples on a 6% denaturing polyacrylamide sequencing gel. Differentially displayed cDNA fragments were excised from the sequencing gel and reamplified using the same primers used in the original PCR reaction. The amplified cDNAs were cloned into a T-tailed pBluescript vector. The Gene Trapper Kit (LifeTechnologies) was used to obtain a larger cDNA fragment of the chMRP gene from a cDNA library constructed from withdrawn chick oviduct mRNA using a cDNA library kit (LifeTechnologies). The chick withdrawn oviduct

cDNA library was screened using the Gene Trapper kit with an oligonucleotide primer (5'-CTGGCCACCCCTATAGCTGC-3') designed from the differential display cDNA clone. cDNA clones were sequenced on an ABI DNA sequencer in the Institute of Human Genetics Microchemical Facility at the University of Minnesota.

RNA isolation and northern blot hybridization. Oviducts were removed from chicks, and the RNA was isolated using RNAzol (TelTest). Total RNA (20µg/lane) was electrophoresed in a 0.8% agarose/formaldehyde gel (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, pH 7.0, and 2.2 M formaldehyde) and transferred to Nytran membrane (Schleicher and Schuell) by capillary transfer. The blots were UV crosslinked using a Stratalinker (Stratagene) and prehybridized at 42°C for a minimum of 3 hours in 50% formamide, 6X SSC, 0.5% SDS, 5X Denhardt's and 100 µg/ml salmon testes DNA. Random labeled (Stratagene) cDNA probes labeled with ^{32}P-dCTP were added to the prehybridization solution, and the blots were hybridized for 16 hours at 42°C in a rotating hybridization oven. The blots were washed at room temperature for 15 minutes in 2X SSC and 0.1% SDS, followed by 15 minutes at 42°C in 2X SSC and 0.1% SDS, and then for 15 minutes at 42°C in 0.2X SSC and 0.1% SDS, and then put on film. The cDNA probe for *chMRP* used in northern blot analysis was the 266 bp DD-PCR product isolated by cutting the chMRP-BS clone with either *EcoR* I or *Rsa* I followed by gel isolating the DNA fragment using a Qiagen gel isolation kit. 18S RNA probes were generated from a mouse 18S RNA clone obtained from American Type Tissue Collection.

Results

Partial cloning and sequence of the chick multidrug resistance-associated protein (*chMRP*) gene. In an effort to understand the regulation of genes involved in chick oviduct development, differential display (DD) was used to clone genes that are down-regulated by estrogen. Sexually immature chicks were implanted with DES, a synthetic estrogen, pellets for at least two weeks prior to DD analysis to initiate oviduct growth and development. After the two weeks of initial DES stimulation, the DES pellets were removed for five days from the chicks that are designated as "withdrawn" (W/D). The chicks

in which the DES pellets were not removed are designated as "stimulated" (Stim). DD was performed using total RNA isolate from withdrawn and stimulated chick oviducts using an oligo $dT_{11}N$ primer and a single random primer. cDNA bands generated by the DD-PCR technique were compared between the estrogen-withdrawn and -stimulated RNA samples. cDNAs that appeared to be down-regulated by estrogen were isolated, re-amplified, subcloned, and sequenced. Sequence analysis of a 266 base pair DD-PCR clone that was differentially expressed in withdrawn and stimulated oviducts showed 73% identity at the nucleotide level with the human multidrug resistance-associated protein (*huMRP*) gene. To further confirm that the DD-PCR clone was in fact the chick homologue of *huMRP*, a larger 1322 bp clone (Figure 1) was obtained by screening an estrogen-withdrawn chick oviduct cDNA library. Nucleotide alignment of the *chMRP* cDNA clone and the *huMRP* gene shows 73% identity over the 1322 bp of the *chMRP* clone. Alignment of the translated *chMRP* clone with the human and murine MRP protein indicates a 73% identity and 79% similarity at the amino acid level (Figure 2). The similarity at both the nucleotide and amino acid level between the chMRP cDNA clone and the human and mouse *MRP* genes indicates that the clone is the chicken homologue of the human MRP gene and is part of the superfamily of ABC transmembrane transporters.

Estrogen regulation of chMRP To confirm that chMRP is indeed differentially expressed in estrogen-stimulated and estrogen-withdrawn chicks, northern blot analysis was carried out using total RNA isolated from chick oviduct (Figure 3). RNA isolated from withdrawn chick oviduct shows strong hybridization of the chMRP DD-PCR probe to an mRNA species of approximately 6.6 kb. In contrast, the stimulated oviduct lane shows a dramatic decrease in the hybridization signal relative to the withdrawn lane.

To determine whether estrogen regulates chMRP mRNA acutely, withdrawn chicks were injected with 17ß-estradiol and, at the indicated times, the oviducts were removed and RNA isolated. As shown by northern blot analysis (Figure 4A and 4B), chMRP mRNA is abundant in the estrogen-withdrawn oviduct (time 0 hour) but rapidly decreases by thirty minutes after injection of estrogen. Densitometric analysis of the northern blots indicates that the chMRP mRNA levels decrease by 70% within one hour, to a level equal to that is observed with chronic estrogen administration (Figure 4B). These results indicate that the

chMRP gene is down-regulated in chick oviduct and that the decrease in chMRP mRNA occurs acutely, within one hour of estrogen administration.

Tissue distribution of chMRP To determine whether chMRP is present in other tissues in the chick, northern blot analysis was used to determine chMRP levels in RNA isolated from estrogen-withdrawn oviduct, liver, brain, heart, kidney, lung, skeletal muscle, and intestine (Figure 5A and 5B). The results demonstrate that chMRP mRNA is most abundant in oviduct, heart, and lung but is also expressed, albeit at about 75% lower levels, in brain, skeletal muscle, kidney, and intestine. chMRP mRNA was not detectable on our northern blots in liver. These results are comparable to those found with murine MRP, which is abundant in lung, heart, kidney, testes, and muscle and is also expressed in very low levels in the liver (Stride et al., 1996).

Discussion

Using the technique of differential display, a partial clone was isolated corresponding to the chick multidrug resistance associated protein gene, *chMRP* (Figure 1). Sequence analysis indicates that the *chMRP* cDNA clone is a homologue of the previously cloned human and murine *MRP* genes (Figure 2) (Cole et al., 1992; Stride et al., 1996). Over the length of the 1322 bp *chMRP* clone, there is significant homology at both the nucleotide and amino acid level with the human and murine *MRP* genes. While the human and murine *MRP* genes are 88% identical at the amino acid level, the *chMRP* clone encodes a peptide that is 79% identical to human and murine MRP (Stride et al., 1996). Structurally, MRP belongs to the family of ATP-binding cassette transmembrane transporter proteins. The members of this family consist of integral membrane ATP-binding proteins that transport a variety of molecules (Higgins, 1992). The region of the *chMRP* gene that was cloned is located between the two nucleotide binding domains. Although members of the ABC transmembrane transporter superfamily share structural and functional similarity, identity at the primary sequence level is low and most of the sequence similarity resides in the nucleotide binding domains. Within the ABC transmembrane transporter superfamily, MRP is most closely related to the *YCF1* gene (43% amino acid identity) (Szczypka et al., 1994) but also has sequence identity with the cystic fibrosis transmembrane regulator (19%) (Riordan et al., 1989), the rat ß cell

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sulfonylurea receptor (29%)(Aguilar-Bryan et al., 1995), and the *ltpgpA* gene (32%) (Callahan and Beverley, 1991; Papadopoulou et al., 1994). The high degree of identity between the *chMRP* clone and the human and murine *MRP* genes indicates that it is indeed the chicken MRP homologue rather than a new member of the ABC transporter superfamily.

These studies also indicate that the *chMRP* gene is acutely down-regulated by estrogen in the chick oviduct. Estrogen is critical for the development and differentiation of the chick oviduct and is involved in the activation of gene expression in cells that express estrogen receptor. However, control of gene expression and oviduct development may also include the down-regulation of genes by estrogen. As shown in Figure 4, an early response to the administration of estrogen is the down-regulation of the amount of chMRP mRNA. Regulation of other members of the ABC transmembrane transporter family by steroids has been shown. Mouse MDR mRNA is increased by estrogen and progesterone in uterine epithelial cells (Arceci et al., 1990; Kuo et al., 1995), and expression of the cystic fibrosis transmembrane regulator gene is also increased by estrogen in uterine epithelial cells (Rochwerger and Buchwald, 1993; Rochwerger et al., 1994). However, our results are the first to show down-regulation of a member of the ABC transmembrane transporter family by steroids. The rapid decrease in chMRP mRNA observed may be due to the decrease in gene transcription or to the decreased stability of the mRNA. Interestingly, in uterine epithelial cells estrogen increased the stability of the MDR mRNA (Kuo et al., 1995). This indicates that estrogen has an effect on both the transcription rate and stability of the mouse MDR mRNA. Although estrogen may decrease the rate of transcription of the *chMRP* gene in chick oviduct, the extremely rapid decrease in chMRP mRNA suggests that there is also an estrogen-induced decrease in its stability as well.

The promoters for the *MRP* gene (Zhu and Center, 1994; Zhu and Center, 1996) and the *mdr* gene (Chen et al., 1990; Madden et al., 1993; Ueda et al., 1987; Ueda et al., 1987) were cloned. Both the *MRP* and *mdr1* genes lack distinctive TATA box elements and have multiple sites of transcriptional initiation. The transcription factor Sp1 is involved in the regulation of both genes (Cornwell and Smith, 1993; Zhu and Center, 1996). However, all the regulatory elements necessary or important for expression of *MRP* have yet to be determined. Since the oviduct is one of the major estrogen-responsive tissues in the chick, another being the liver, it is probable that in the chicken MRP is

regulated by estrogen in a tissue-specific manner.

Our results indicate that the *chMRP* gene is rapidly down-regulated by estrogen in the chick oviduct. While there is evidence from *in vitro* studies that MRP can confer the MDR phenotype, it is not clear what role MRP plays in the MDR phenotype in tumors (Grant et al., 1994). Determination of the elements required for activation or repression of *MRP* would be beneficial in understanding and perhaps treating tumors that exhibit the multidrug resistant phenotype, especially those that are responsive to estrogen..

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FIGURE LEGENDS

FIGURE 1

Partial cloning of the chicken multidrug resistance-associated protein gene. Differential display-PCR was used to clone a fragment of the *chMRP* gene that is differentially expressed between estrogen-stimulated chicks and -withdrawn chicks. Additional sequence of the *chMRP* gene was cloned using the GeneTrapper kit (LifeTechnologies). A BLAST search indicated that the 1332 bp of the *chMRP* gene that was cloned aligns with the human MRP sequence.

FIGURE 2

Alignment of chMRP amino acid sequence with human and murine MRP. The 1332 bp of the *chMRP* clone were translated and aligned with the human MRP (humMRP) and murine MRP (murMRP) proteins. The human and murine MRP amino acid sequences are 73% identical to the chMRP sequence. Numbering corresponds to the cloned chMRP fragment. The huMRP and murMRP sequences are from amino acids 832 to 1277 and 832 to 1272, respectively.

FIGURE 3

ChMRP mRNA is down-regulated by estrogen in the chick oviduct. Northern blot analysis was carried out using 20 µg total RNA isolated from estrogen-withdrawn (W/D) and estrogen-stimulated (STIM) chick oviduct. The 266 bp *chMRP* differential display cDNA clone was used as the probe. The lower panel shows the 28s and 18s rRNA bands stained with EtBr to indicate RNA loading. The chMRP probe hybridizes to a 6.6 kb band that is more abundant in the estrogen-withdrawn oviduct lane than the estrogen-stimulated oviduct lane.

FIGURE 4

chMRP mRNA expression is acutely down-regulated in the oviduct by estrogen. To determine whether the estrogen-induced decrease in chMRP mRNA occurs acutely, chicks that had the DES pellets withdrawn for five days were injected intravenously with 25 mg/kg 17-ß-estradiol. Oviducts were harvested at the indicated times (0, 0.5, 1, 2, and 4 hours) and total RNA isolated. A.) Northern blot analysis was carried out using the *chMRP* clone as a probe and an 18s rRNA probe was used as a correction for sample loading. B.) A plot of the chMRP/18s rRNA values indicates that chMRP mRNA decreases rapidly after the injection of 17-ß-estradiol. chMRP mRNA levels decrease by 50% within 30 minutes and by 70% within one hour, which is comparable to

levels observed with chronic estrogen administration.

FIGURE 5

***chMRP* gene expression varies between tissues.** Tissue distribution of chMRP mRNA was determined by northern blot analysis using tissues from chicks withdrawn from estrogen for five days. A.) The northern blot was probed with the *chMRP* clone and an 18s rRNA probe. B). The chMRP mRNA/18s rRNA values were determined by densitometry. chMRP mRNA is most abundantly expressed in the chick oviduct, heart, and lung. The brain, skeletal muscle, kidney, and intestine show lower levels of chMRP mRNA expression. chMRP mRNA was not detected in the liver.

```
chMRP   .................................................CGCGGC  6
                                                         || ||
humMRP  GAAGAACAAGACGCGGATCTTGGTCACGCACAGCATGAGCTACTTGCCGC  2500

chMRP   CGCGTCGACCAATTCTGGTAATGACTGATGGAGAAATCTCTGAGATGGGC  56
          |        ||  | || |||| ||  ||  | |||||||||||||||
humMRP  AGGTGGACGTCATCATCGTCATGAGTGGCGGCAAGATCTCTGAGATGGGC  2550

chMRP   TCCTACCAGGAGCTGCTGAAGCAGGATGGAGCTTTTGCAGAGTTCCTTCG  106
        |||||||||||||||||||    |  || || || || || |||||||| ||
humMRP  TCCTACCAGGAGCTGCTGGCTCGAGACGGCGCCTTCGCTGAGTTCCTGCG  2600

chMRP   TACGTACGCTAATGCTGAACA....AAGCATGGAGAGCAGTGATGCAAGT  152
        ||| || || |   | || ||      || ||| |||| ||    |
humMRP  TACCTATGCCAGCACAGAGCAGGAGCAGGATGCAGAGGAGAACGGGGTCA  2650

chMRP   AGTCCAT........CTGGAAAGGAACGAAAGCCCGTAGAAAATGGAGTC  194
         | | ||        | || |||||| ||||| | || ||||| |
humMRP  CGGGCGTCAGCGGTCCAGGGAAGGAAGCAAAGCAAATGGAGAATGGCATG  2700

chMRP   CTTGTGAATGACGCCCCTGGAAAGCTGATGCATCGGCAGCTCAGTAACTC  244
        || ||||  |||   | || |||||    ||||  | ||||||||| | |||
humMRP  CTGGTGACGGACAGTGCAGGGAAGCAACTGCAGAGACAGCTCAGCAGCTC  2750

chMRP   CTCCACATACAGCAGAGAAACTGGGAAGTCACAGCACCAGAGCAGCACAG  294
        |||| | || ||  | || |  | |          |||||| | ||||||| |
humMRP  CTCCTCCTATAGTGGGGACATCAGCA......GGCACCACAACAGCACCG  2794

chMRP   CAGAGCTGCAGAAGCCTCTTGCAGAGA...AGAATTCCTGGAAACTGACA  341
        |||| |||||||||  ||  ||  |||  || | ||||||| ||||
humMRP  CAGAACTGCAGAAAGCTGAGGCCAAGAAGGAGGAGACCTGGAAGCTGATG  2844

chMRP   GAGGCTGACACAGCAGAGACTGGGAGGGTAAAGGCAACAGTATACTGGGA  391
        ||||||||||  ||  ||||| |||  ||| |||     | || |||||||||
humMRP  GAGGCTGACAAGGCGCAGACAGGGCAGGTCAAGCTTTCCGTGTACTGGGA  2894

chMRP   ATACATGAAAGCTATTGGACTCTATATCTCTTTTCTGAGCGTTTTCCTCT  441
         |||||||| ||  || |||||||||  |||||  || ||  ||| | ||||| |
humMRP  CTACATGAAGGCCATCGGACTCTTCATCTCCTTCCTCAGCATCTTCCTTT  2944

chMRP   TTATGTGTAACCATATAGCCTCCCTGGCTTCCAACTACTGGCTAAGTTTA  491
        | ||||||||||||| |   || | ||||||||||||||| |||||| ||  |
humMRP  TCATGTGTAACCATGTGTCCGCGCTGGCTTCCAACTATTGGCTCAGCCTC  2994

chMRP   TGGACAGATGATCCGGTTGTCAATGGGACACAGCAGTACACAAATGTCAG  541
        ||||| |||||| ||  | ||||| ||||| ||| || |||| || ||| |
humMRP  TGGACTGATGACCCCATCGTCAACGGGACTCAGGAGCACACGAAAGTCCG  3044

chMRP   ACTGGGAGTATATGGAGCGCTGGGAATTTCTCAAGGTATTGCTGTGTTTG  591
        ||| | | || |||||||||| ||||| |||||| ||||| || || ||||||||
humMRP  GCTGAGCGTCTATGGAGCCCTGGGCATTTCACAAGGGATCGCCGTGTTTG  3094

chMRP   GCTACTCGATGGCTGTGTCAATAGGAGGAATATTTGCTTCACGGCACCTG  641
        |||||||| ||||| ||||| || || || || || || || ||||| ||   |||
humMRP  GCTACTCCATGGCCGTGTCCATCGGGGGGATCTTGGCTTCCCGCTGTCTG  3144
```

FIGURE 1

```
chMRP    CACCTCGACCTGCTGCACAATGTTCTCAGGTCTCCAATGAGTTTCTTTGA  691
         ||| | ||||||||||||||  | ||  |||| || ||||| ||||||||
humMRP   CACGTGGACCTGCTGCACAGCATCCTGCGGTCACCCATGAGCTTCTTTGA  3194

chMRP    ACGTACACCCAGTGGAAATTTAGTGAACCGTTTCTCTAAGGAGATAGATA  741
          || || |||||||| ||  |  ||||||||| |||||| |||||| | || |
humMRP   GCGGACCCCCAGTGGGAACCTGGTGAACCGCTTCTCCAAGGAGCTGGACA  3244

chMRP    CCATTGACTCTACCATTCCACCAATCATCAAGATGTTCATGGGCTCAACA  791
         |  | |||||| |  || ||       |||||||||||||||||||||||||
humMRP   CAGTGGACTCCATGATCCCGGAGGTCATCAAGATGTTCATGGGCTCCCTG  3294

chMRP    TTTAACGTGATTGGGGCTTGTATCATCATTTTGCTGGCCACCCCTATAGC  841
          ||  |||||| |||||| ||  || ||| | ||  |||||||||| || || ||
humMRP   TTCAACGTCATTGGTGCCTGCATCGTTATCCTGCTGGCCACGCCCATCGC  3344

chMRP    TGCTGTCGTTATTCCACCTCTGGGACTTGTCTACTTGCTTGTGCAGAGAT  891
          ||   || | || || || || || || ||  |||||||  | || ||||| |
humMRP   CGCCATCATCATCCCGCCCCTTGGCCTCATCTACTTCTTCGTCCAGAGGT  3394

chMRP    TTTATGTGGCCACTTCTCGCCAGCTCAAACGCCTTGAATCTGTTAGTCGT  941
          | || |||||     | || || ||||| ||  ||||| || || || || ||
humMRP   TCTACGTGGCTTCCTCCCGGCAGCTGAAGCGCCTCGAGTCGGTCAGCCGC  3444

chMRP    TCTCCTGTGTATTCTCACTTCAATGAGACCCTTCTGGGAGTCAGTGTAAT  991
          || || || ||||| || ||||| | ||||| |||||| ||||| || ||
humMRP   TCCCCGGTCTATTCCCATTTCAACGAGACCTTGCTGGGGGTCAGCGTCAT  3494

chMRP    TCGAGCCTTTGAGGAACAGAAACGTTTTATAAAGCAGAATGACATGAAAG  1041
          |||||||||| |||||  ||| |  || || ||  | |||| |||| ||||| |
humMRP   TCGAGCCTTCGAGGAGCAGGAGCGCTTCATCCACCAGAGTGACCTGAAGG  3544

chMRP    TGGATGAAAATCAGAAAGCTTATTACCCAAGCATTGTTGCAAACAGATGG  1091
          ||||  ||  || ||||| ||  ||||||||| |||||  || ||||| |||
humMRP   TGGACGAGAACCAGAAGGCCTATTACCCCAGCATCGTGGCCAACAGGTGG  3594

chMRP    CTGGCAGTACGTCTGGAGTTTGTGGGGAACTGTATTGTTCTCTTTGCAGC  1141
          ||||| || ||  ||||||| |||||| ||||| ||||  || ||||| ||
humMRP   CTGGCCGTGCGGCTGGAGTGTGTGGGCAACTGCATCGTTCTGTTTGCTGC  3644

chMRP    ATTGTTTGCAGTGATTGCACGCAACAAGCTCAGTCCGGGACTGATTGGTC  1191
           ||||||| |||||   |  |   ||| ||||| | ||  || | || |
humMRP   CCTGTTTGCGGTGATCTCCAGGCACAGCCTCAGTGCTGGCTTGGTGGGCC  3694

chMRP    TTTCAGTGTCCTATTCGCTGCAGATTACAGCATACTTAAACTGGCTAGTT  1241
          | |||||||| || || ||  ||||| | || | |||||| ||||||| |||
humMRP   TCTCAGTGTCTTACTCATTGCAGGTCACCACGTACTTGAACTGGCTGGTT  3744

chMRP    CGTATGACATCTGATCTGGAAACCAACATTGTTGCTGTAGAAAGAGTCAA  1291
          || |||| |||||   ||||||||||||||| || || || || ||  ||||
humMRP   CGGATGTCATCTGAAATGGAAACCAACATCGTGGCCGTGGAGAGGCTCAA  3794

chMRP    AGAATATGCTGAAATGGAGAAGGAGGTACAG..................  1322
          || ||| | || |  |||||||||||  |
humMRP   GGAGTATTCAGAGACTGAGAAGGAGGCGCCCTGGCAAATCCAGGAGACAC  3844
```

FIGURE 1 (cont.)

```
         801                                                     850
  chMRP                                            RPRRPIL VMTDGEISEM
  huMRP  AHVGKHIFEN VIGPKGMLKN KTRILVTHSM SYLPQVDVII VMSGGKISEM
 murMRP  AHVGKHIFEK VVGPMGLLKN KTRILVTHGI SYLPQVDVII VMSGGKISEM

         851                                                     900
  chMRP  GSYQELLKQD GAFAEFLRTY ANAEQSMESS DAS....SPS GKERKPVENG
  huMRP  GSYQELLARD GAFAEFLRTY ASTEQEQDAE ENGVTGVSGP GKEAKQMENG
 murMRP  GSYQELLDRD GAFAEFLRTY ANAEQDLASE DD...SVSGS GKESKPVENG

         901                                                     950
  chMRP  VLVNDAPGKL MHRQLSNSST YSRETGKSQH QSSTAELQKP LAEK.NSWKL
  huMRP  MLVTDSAGKQ LQRQLSSSSS YSGDI..SRH HNSTAELQKA EAKKEETWKL
 murMRP  MLVTDTVGKH LQRHLSNSSS HSGDT..SQQ HSSIAELQKA GA.KEETWKL

         951                                                    1000
  chMRP  TEADTAETGR VKATVYWEYM KAIGLYISFL SVFLFMCNHI ASLASNYWLS
  huMRP  MEADKAQTGQ VKLSVYWDYM KAIGLFISFL SIFLFMCNHV SALASNYWLS
 murMRP  MEADKAQTGQ VQLSVYWNYM KAIGLFITFL SIFLFLCNHV SALASNYWLS

         1001                                                   1050
  chMRP  LWTDD.PVVN GTQQYTNVRL GVYGALGISQ GIAVFGYSMA VSIGGIFASR
  huMRP  LWTDD.PIVN GTQEHTKVRL SVYGALGISQ GIAVFGYSMA VSIGGILASR
 murMRP  LWTDDPPVVN GTQANRNFRL SVYGALGILQ GAAIFGYSMA VSIGGIFASR

         1051                                                   1100
  chMRP  HLHLDLLHNV LRSPMSFFER TPSGNLVNRF SKEIDTIDST IPPIIKMFMG
  huMRP  CLHVDLLHSI LRSPMSFFER TPSGNLVNRF SKELDTVDSM IPEVIKMFMG
 murMRP  RLHLDLLYNV LRSPMSFFER TPSGNLVNRF SKELDTVDSM IPQVIKMFMG

         1101                                                   1150
  chMRP  STFNVIGACI IILLATPIAA VVIPPLGLVY LLVQRIYVAT SRQLKRLESV
  huMRP  SLFNVIGACI VILLATPIAA IIIPPLGLIY FFVQRFYVAS SRQLKRLESV
 murMRP  SLFSVIGAVI IILLATPIAA VIIPPLGLVY FFVQRFYVAS SRQLKRLESV

         1151                                                   1200
  chMRP  SRSPVYSHFN ETLLGVSVIR AFEEQKRFIK QNDMKVDENQ KAYYPSIVAN
  huMRP  SRSPVYSHFN ETLLGVSVIR AFEEQERFIH QSDLKVDENQ KAYYPSIVAN
 murMRP  SRSPVYSHFN ETLLGVSVIR AFEEQERFIH QSDLKVDENQ KAYYPSIVAN

         1201                                                   1250
  chMRP  RWLAVRLEFV GNCIVLFAAL FAVIARNKLS PGLIGLSVSY SLQITAYLNW
  huMRP  RWLAVRLECV GNCIVLFAAL FAVISRHSLS AGLVGLSVSY SLQVTTYLNW
 murMRP  RWLAVRLECV GNCIVLFAAL FAVISRHSLS AGLVGLSVSY SLQITAYLNW

         1251                                                   1300
  chMRP  LVRMTSDLET NIVAVERVKE YAEMEKE
  huMRP  LVRMSSEMET NIVAVERLKE YSETEKEAPW QIQETRPPSS WPQVGRVEFR
 murMRP  LVRMSSEMET NIVAVERLKE YSETEKEAPW QIQETAPPST WPHSGRVEFR
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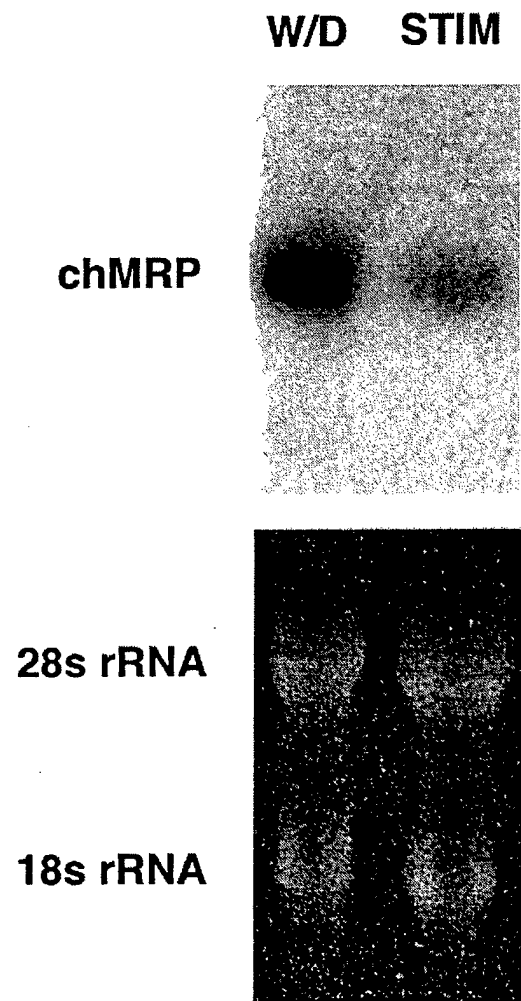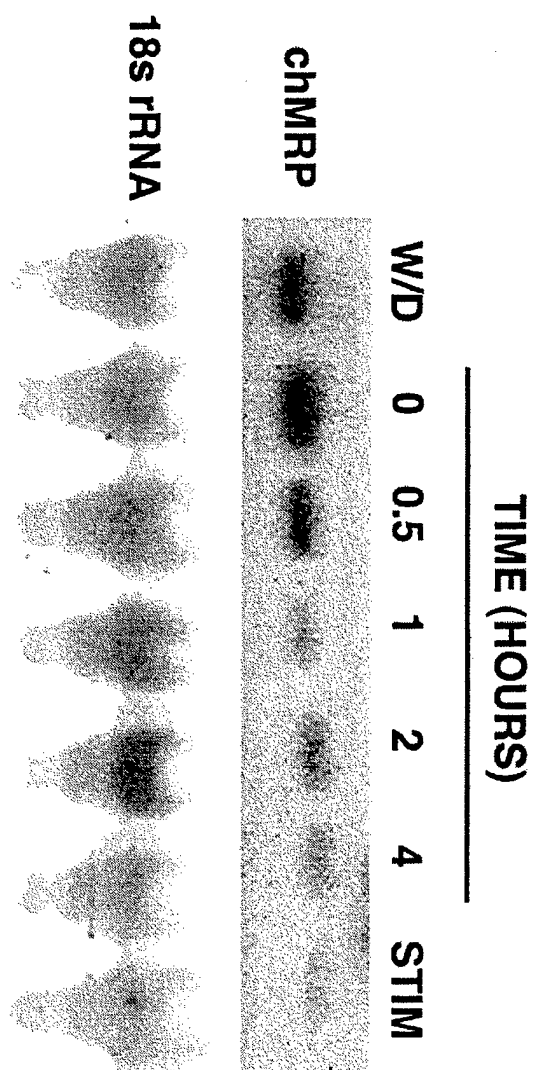
FIGURE 2

W/D   STIM
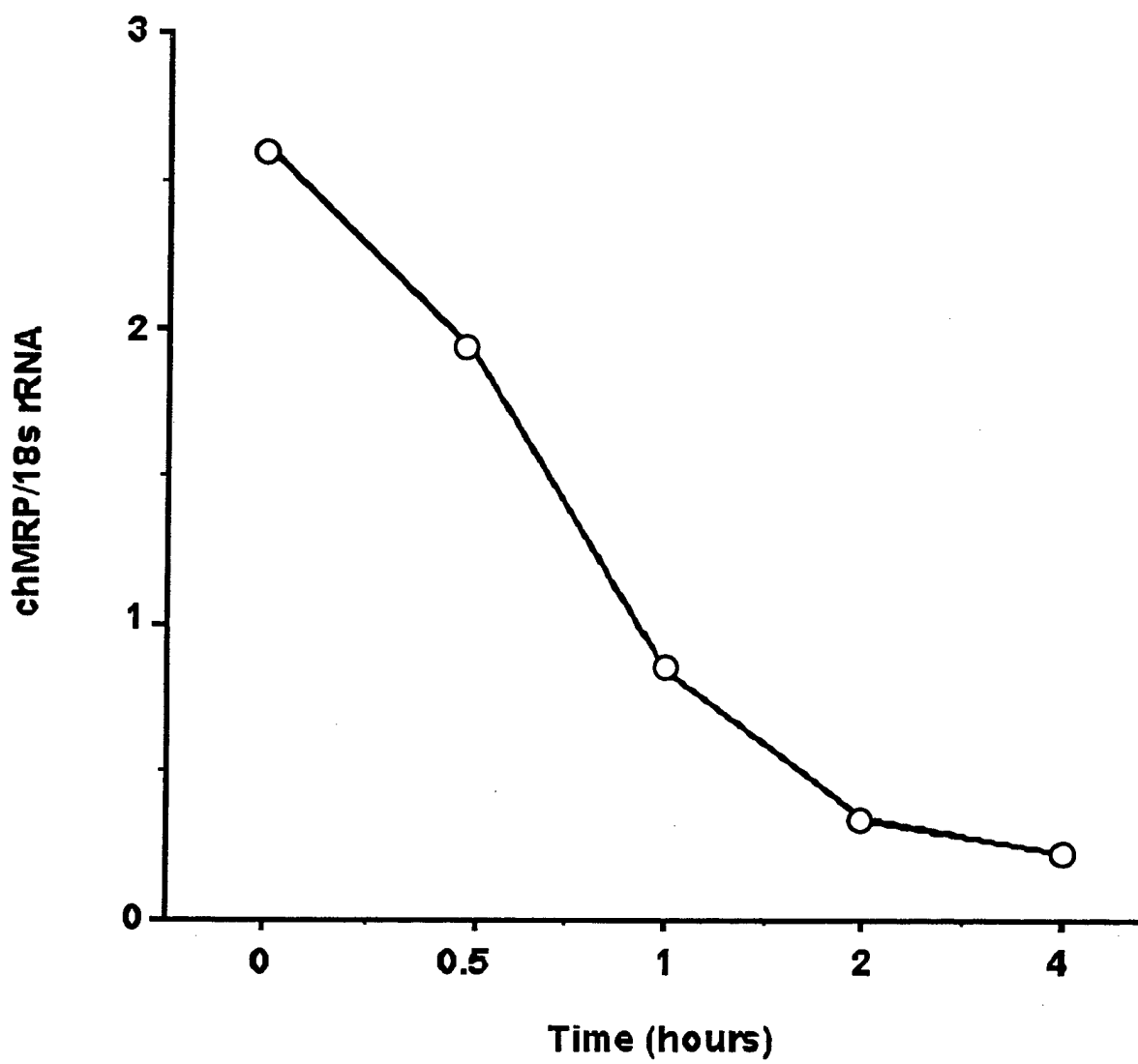
chMRP

28s rRNA

18s rRNA

FIGURE 3

FIGURE 4A

FIGURE 4B
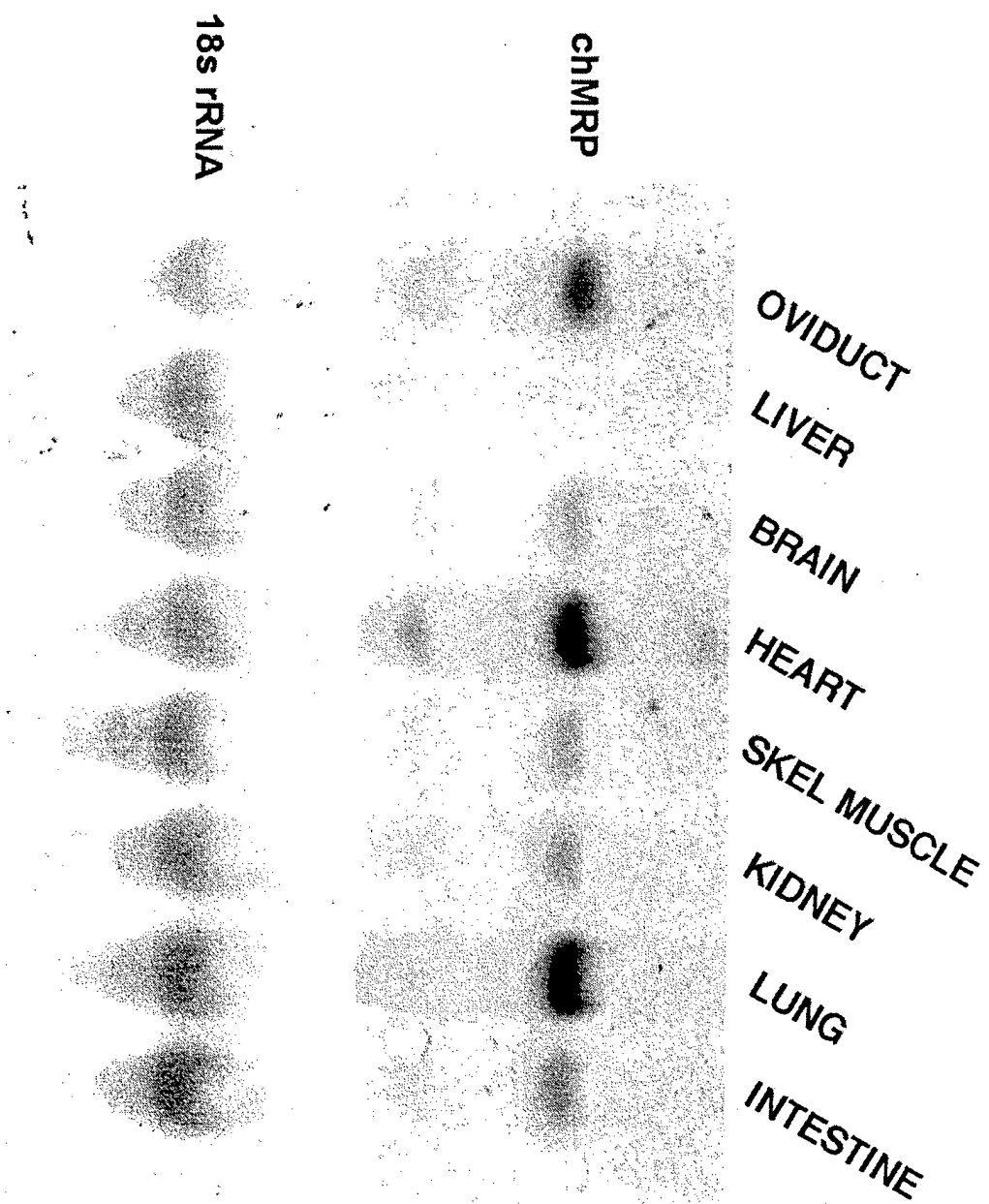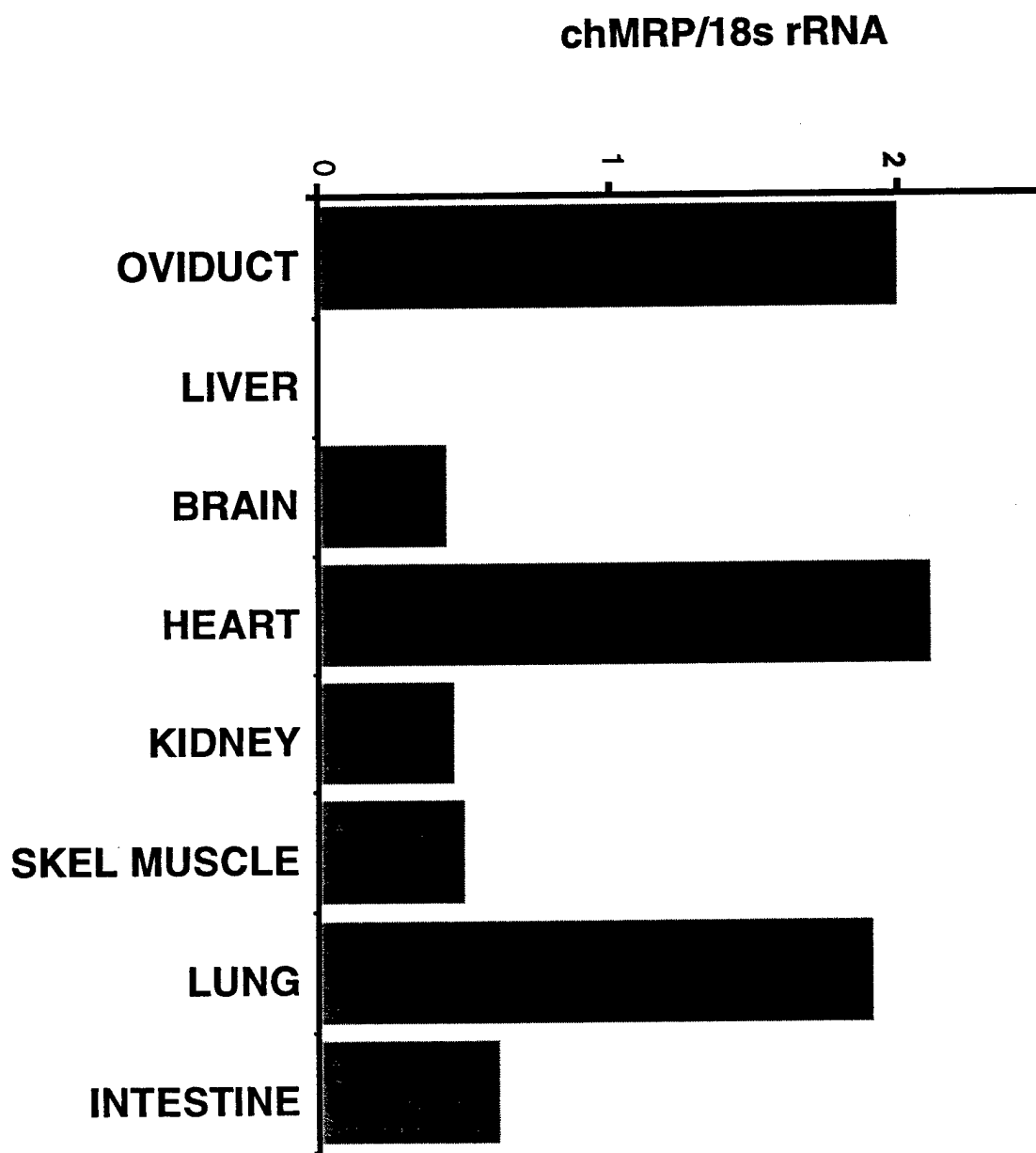
18s rRNA

chMRP

OVIDUCT
LIVER
BRAIN
HEART
SKEL MUSCLE
KIDNEY
LUNG
INTESTINE

FIGURE 5A

chMRP/18s rRNA

FIGURE 5B

APPENDIX B

chMRP

18s rRNA
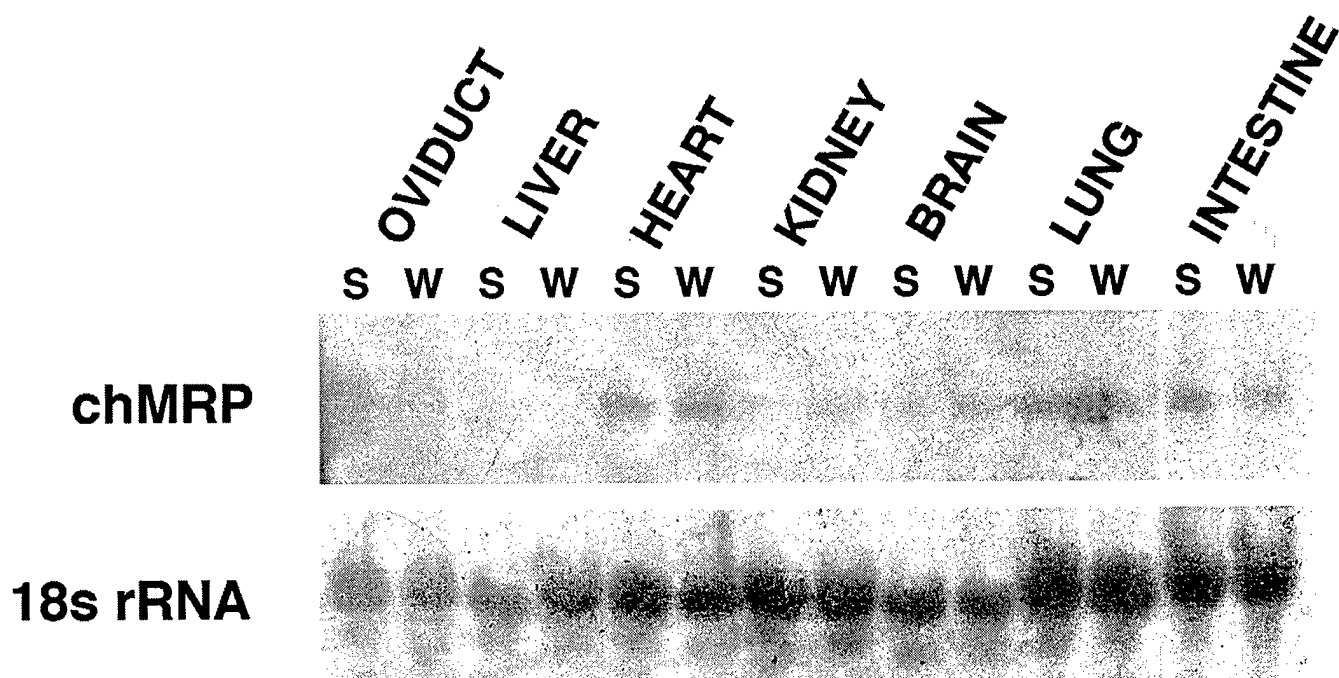
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   1  GCTGGGCAAA GATGAGCTTA TTGACTACAT CTGCAGCGAT GAACTGGTGA

  51  TCACGAAGGA AGAGATGGTG TTCGAAGCTG TCATGCGCTG GGTGTACCGG

 101  GCGGTCGAGC TGCGAAGACC GGTGTTACAC GAGCTGCTGA CGCACGTCAG

 151  GCTCCCGTTG TTACACCCCA ACTACTTCGT TCAGACTGTG GAGGTGGACC

 201  AGCTGATTCA GAATTCCCCA GAGTGCTATC AGCTGCTGCA CGAGGCCAGG

 251  AGGTACCATA TCCTTGGCAA TGAGATGATG TCCCCCAGGA CTCGGCCACG

 301  CAGATCAACT GGTTATTCTG AGGTGATAGT TGTTGTTGGA GGCTGTGAAA

 351  GAGTTGGAGG GTTTAACTTG CCATACACTG AGTGCTACGA TCCTGTAACA

 401  GGAGAATGGA AATCACTGGC CAAACTTCCA GAGTTTACCA AGTCTGAGTA

 451  TGCAGTGTGT GCCCTACGGA ATGATATCCT TGTTTCAGGT GGAAGAATCA

 501  ATAGTCGGGA TGTATGGATT TATAACTCTC AGCTTAACAT TTGGATCAGA

 551  GTTGCCTCCT TGAATAAAGG CAGATGGCGT CATAAAATGG CTGTTCTTCT

 601  TGGTAAAGTG TATGTCGTTG GAGGGTATGA TGGGCAAAAC CGCCTCAGCA

 651  GCGTGGAGTG CTACGATTCG TTTTCCAATC GATGGACAGA GGTGGCTCCC

 701  CTCAAGGAAG CCGTGAGCTC TCCTGCAGTC ACCAGCTGTG TCGGCAAACT

 751  GTTTGTGATC GGGGGCGGTC CTGATGACAA CACGTGTTCT GACAAGGTTC

 801  AGTCTTATGA TCCTGACACT AATTCTTGGC TGCTCCCTGC CACTATCCCT

 851  ATTGCAAAAA GATGTATTAC GGCTGTGTCT TTAAACAATC TGATTTATGT

 901  TGCTGGTGGG CTCACCAAGG CAATATACTG CTATGATCCA ATTGAGGACT

 951  AACTGGATGC ATGTACAGAA TAACGTTCAG CAGACAGGAG AATTGTGGCA

1001  TGTCTGTGTG TAATGGAAAG ATCTATATCC TTGGTGGAAG ACGAGAAAAC

1051  GGTGAAGCCA CAGACACTAT TCTTTGCTAT GATCCTGCAA CGGGCATTAT

1101  CACAGGAGTA GCAGCCATGC CCAGGCCAGT ATCGTATCAT GGCTGTGTGA

1151  CCATTCATAG GTATAATGAA AAAGGCTTTA AACTGTAATT TGTTTTGGAG

1201  GAGAAGAAAA GGAGAAGATG ATGGCATGAA TGAATCCAGT GGTCTGCTGC

1251  AAGACTGATT TATAAATCCG AAAGTGGGAA AATGCCCTTT CCCTGCCTAA

1301  GTGTCATAAA AAATTGTAYC CTGTGCCTTT TTGGGGAGAG GAAAAGAAAA

1351  AAAAAAAAAA AAA
```
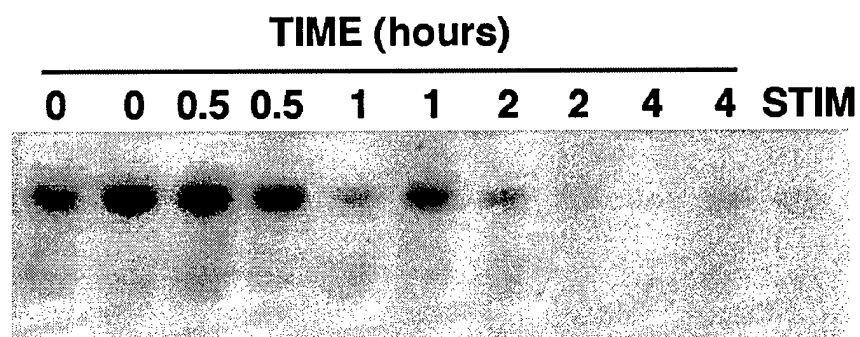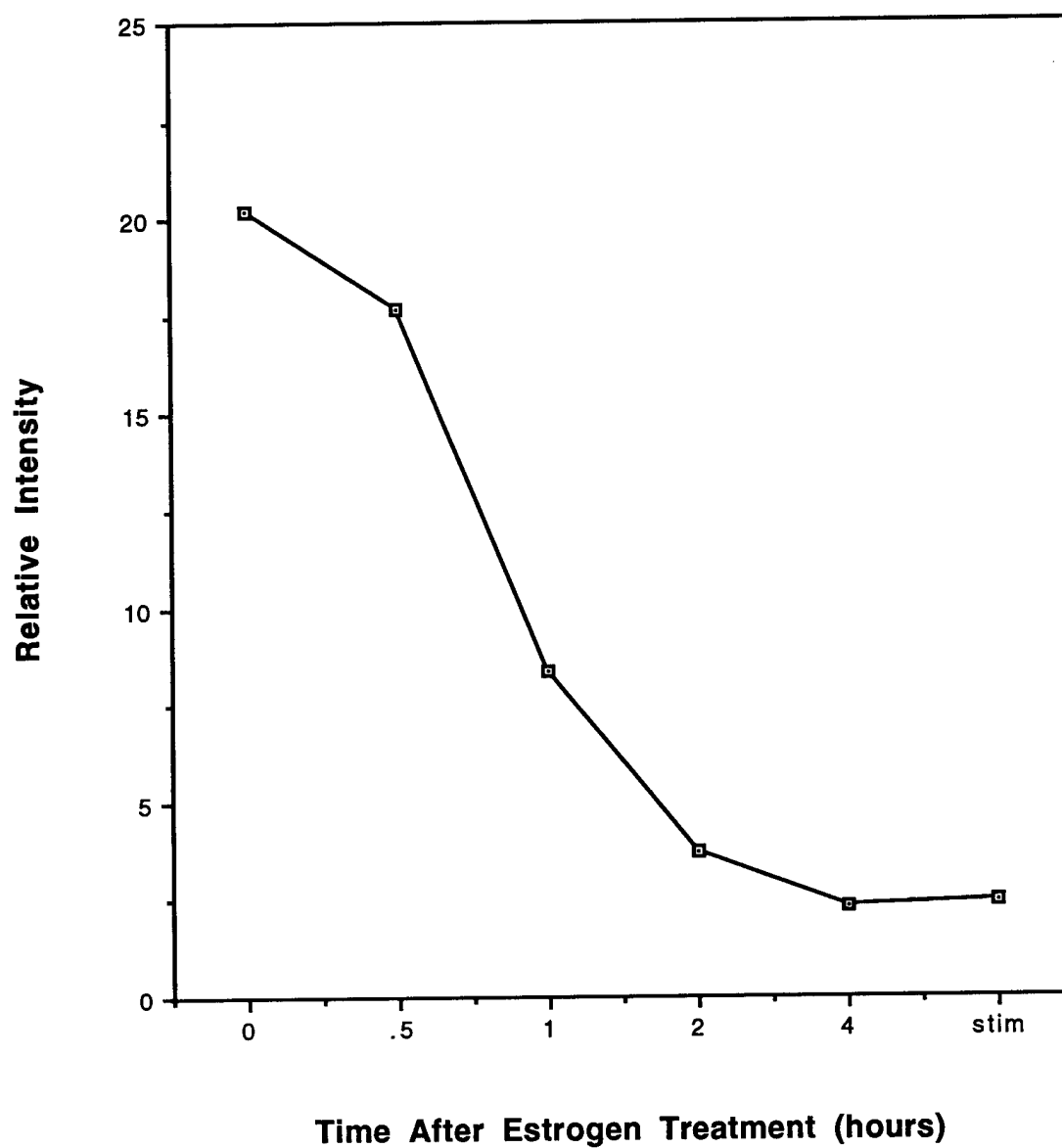
Repression of Clone #41 mRNA by Estrogen

TIME (hours)

| 0 | 0 | 0.5 | 0.5 | 1 | 1 | 2 | 2 | 4 | 4 | STIM |

Figure 3

Time Course of Repression of Clone #41 mRNA by Estrogen

Time After Estrogen Treatment (hours)

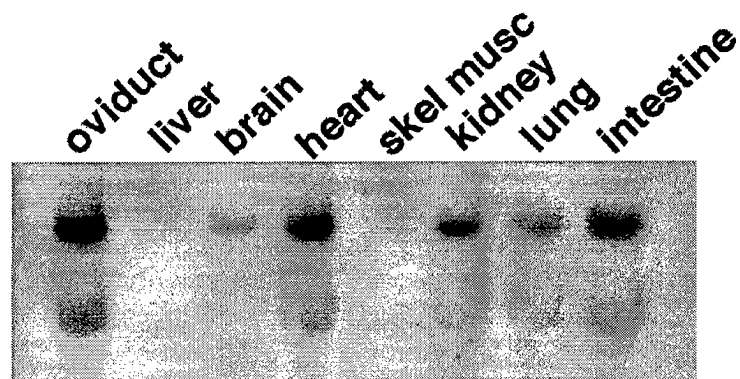# Tissue Distribution of Clone #41 mRNA

Figure 5

Relative Distribution of the Two Clone #41 mRNAs in Different Tissues